Establishment of Axenic Culture and Induction of Organogenesis in Kapur Bukit (*Dryobalanops beccarii* Dyer)

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LIST OF ABBREVIATONS

PGRs	Plant Growth Regulators
2, 4-D	2, 4-Dichlorophenoxy Acetic Acids
NAA	α- Naphthalene Acetic Acid
BA	Benzyladenine
BAP	6-Benzlaminopurine
TDZ	Thidiazuron
IAA	Indole-3-acetic Acid
IBA	Indole-3- butyric Acid
MS	Murashige and Skoog (1962)
WPM	Woody Plant Medium (McCown & Llyod, 1981)
PPM	Plant Preservative Mixture
PVP	Polyvinyl Pyrolidone
TET	Tetracycline
CRD	Completely Randomized Design
ANOVA	Analysis of Variance

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ABSTRACT

Dryobalanops beccarii Dyer (Kapur Bukit) has been recommended by Sarawak Forest Department as one of the four timber species for forest plantation in Sarawak (Thai, 2002) and clonal materials are preferred for this purpose. However, the production of large quantity of planting stock through conventional propagation has not been very successful as Kapur Bukit posses recalcitrant seed and is irregular in flowering and thus fruiting. The *in vitro* technique therefore has been considered as an alternative for mass production of clonal material. Nevertheless, previous studies had shown that, it was difficult to establish aseptic and viable explants in culture because of browning and contamination. In 2009, Ang has found that phenolic released from petiolule explants were negligible. Roots also were successfully induced from friable callus that derived from this explants. To improve the percentage of axenic and viable explants in culture, further research was done by modifying the disinfection protocol that previously developed. Organogenesis of Kapur Bukit also was carried out by using various types and concentration of cytokinins, either alone or in combination with auxin to induce shoot formation. From this study, it was found that organogenesis of root was successfully induced at concentration 0.5, 1.0, 1.5, 2.0 mg/L of 2, 4-D. While green callus was observed in 4 mg/L BAP, 2 mg/L BAP+ 1 mg/L Kin and 3 BAP+ 0.5 mg/L Kin supplemented WPM medium. However, no sign of shoot formation was observed as phenolic that released caused green callus died after 3 months cultured. Seems browning problem is more critical than microbial contamination.

Key words: browning, organogenesis, petiolule explants

ABSTRAK

Dryobalanops beccarii Dyer (Kapur Bukit) telah dikenalpasti oleh Pertubuhan Perhutanan Sarawak sebagai salah satu daripada spesis balak untuk perladangan hutan di Sarawak dan bahan bakaan adalah merupakan pilihan utama untuk tujuan ini. Walaubagaimanapun, penghasilan stok kuantiti yang besar melalui propagasi vegetatif konvesyen tidak begitu berjaya kerana spesis ini jarang menghasilkn bunga dan juga memiliki biji benih yang bandel. Teknik in vitro telah dipertimbangkan sebagai teknik alternatif untuk menghasilkan bahan bakaan. Walaubagaimanapun, kajian sebelum ini menunjukkan bahawa ianya adalah sukar untuk mendapatkan ekplan yang aseptik dan berhidup kerana wujudnya masalah kontaminasi dan keperangan. Pada tahun 2009, Ang telah mendapati bahawa penghasilan phenol adalah kurang daripada ekplan tangkai. Akar juga berjaya diinduksikan daripada kalus gampang pecah dari ekplan ini. Untuk meningkatkan peratus kultur berhidup, kajian yang lebih mendalam telah dijalankan dengan mengubah proptokol disinfeksi yang telah dikembangkan sebelum ini. Kajian yang mendalam tentang organogenesis Kapur Bukit juga diteruskan dengan mengunakan beberapa jenis dan kepekatan sitokinin, samada secara sendirian atau digabungkan dengan auxin untuk menginduksi pembentukan pucuk. Daripada kajian ini, pembentukkan akar telah dilihat pada kepekatan 0.5, 1.0, 1.5 dan 2.0 mg/L 2, 4-D. Manakala, kalus hijau telah dilihat pada media WPM yang mengandungi 4 mg/L BAP, 2 mg/L BAP+ 1 mg/L Kin and 3 BAP+ 0.5 mg/L Kin. Walaubagaimanapun, tiada petanda pembentukkan pucuk dilihat daripada kalus hijau kerana phenol yang terbebas menyebabkan kalus ini mati selepas 3 bulan dikultur. Daripada kajian ini, didapati masalah keperangan adalah lebih kritikal daripada masalah kontaminasi mikrobial.

Kata kunci: keperangan, organogenesis, eksplan tangkai

1.0 INTRODUCTION

The dipterocarps are the main species in the tropical forests of Southeast Asia. They play an important role in the economy of many Southeast Asian countries and predominate the international tropical timber market. Non-timber forest product (NATPs) of dipterocarps such as nuts, dammar, resin and camphor also has a large impact on the economies of the rural communities and forest dwellers (Apanah & Turnbull, 1998). *Dryobalanops beccarii* Dyer is one of the dipterocarps species commonly known as Kapur Bukit. It is an important timber for domestic needs in Asia (Apanah & Turnbull, 1998), for timber utilization (Chikaya, 1997) and also for maintaining the forests. However, the demand of timber was exceeding the supply from the natural forest because of over logging as well as the slowing down of forest clearing for agriculture development. According to Kasran (1988) the logging activity is now confined to the hilly areas of dipterocarps forest in Peninsular Malaysia. Consequently, the supply of dipterocarps becomes critical (Thai, 2002). In order to sustain the vitality of wood-based industries, there is a need to look for the alternative source of timber supply other than natural forest. The most important thing is to ensure a steady supply of industrial wood in the future (Apanah & Turnbull, 1998).

The launching of forest plantation through the Compensatory Forest Plantation Project (CFPP) is the obvious option to sustain the supply of timber tree. Establishment of fast growing hardwood plantation based on exotic species in Asia forest have faced many difficulties and there is increasing interest in indigenous species for this effort . Many of the species under consideration are dipterocarps (Appanah & Turnbull, 1998). According to Thai (2002), *D. beccarii* Dyer is one of the species that is under consideration because it is an

indigenous species and fast-growing. There are two common ways to produce large quantity of *D. beccarii* Dyer planting stock for forest plantation purposes. First is through conventional method by seeds and second is by vegetative propagation through stem cutting. However, there is a problem about the stable or continuous supply of seedling because this species flowers irregularly (Appanah & Turnbull, 1998). Another problem of using seedling as the planting material is *D. beccarii* Dyer has recalcitrant seed. According to Tompsett (1994), 94% of dipterocarps species posses a recalcitrant seed. Under moist and warm condition, recalcitrant seed will quickly germinate, therefore difficult to store. While, vegetative propagation by stem cutting is unsatisfactory as the rooting of cutting is very difficult and the stem cutting tends to form plagiotropic growth (Edward, 2005).

The alternative way that has been suggested is micropropagation by *in vitro* culture technique. Ang (2009) and Kwan (2008) have made some progresses in the establishment of the axenic culture of *D. beccarii* Dyer. Nearly 70% contamination-free and viable shoot-tip explants of *D. beccarii* Dyer culture has been successfully produced (Ang, 2009). Therefore, the first objective of this research is to maintain and to improve the percentage of axenic and viable culture of *D. beccarii* Dyer. To begin with, the disinfection procedure that was developed by Ang (2009) was adopted with modification. Up to date, an organogenesis of root from developing petiolule callus had been successfully induced by Ang (2009). Therefore, the second objective of this study is to attempt the organogenesis of shoot either directly from petiolule callus or by petiolule-derived root callus.

2.0 LITERATURE REVIEW

2.1 Botanical Description of *Dryobalanops beccarii* Dyer

Dryobalanops beccarii Dyer (*D. beccarii* Dyer) is a species that belong to Dipterocarpaceae family. The species is named after Odoardo Beccarii, 1843-1920, who is an Italian explorer and a botanist. The synonym name of this species are *Dryobalanops beccariana* Ridl. and *Dryobalanops oocarpa* Sloot. In Borneo, this woody plant is called Kapur Bukit (Sarawak), Kapur Merah (Sabah) and Keladan (Iban). *D. beccarii* Dyer can grow up to 57 m tall and 160 cm in diameter. According to Soerianegara and Lemmens (2001) as cited in Tie (2007), the density of the *D. beccarii* Dyer wood is 600-710 kg/m^3 moisture content. Their stems have resin which is a sticky flammable organic compound which differentiated Dipterocarps from non-Dipterocarp species. The leaf is alternate and simple, while the inflorescence is panicle. The flower is 25 mm in diameter and white in colour. Its fruit 19 mm long, green-yellow-reddish in colour, has five wings with 65 mm long and placed on the calyx cup. These properties allow the pollination by wind. *D. beccarii* has terrestrial system and abundant on leached sandy soils and sometimes in periodical. According to Ashton (2004), *D. beccarii* Dyer is only distributed within Borneo Island (Brunei, Sarawak and Kalimantan) and certain part of Johore in Peninsular Malaysia. In Brunei, *D. beccarii* Dyer has been identified as the best quality *Dryobalanops* compared to other species of the same genus.

2.2 Problem by Seed Propagation and Conventional Methods

A dipterocarp seed has been used in tissue culture quite extensively by many researchers. However, dipterocarps have sporadic seed production, which flowering and fruiting is

irregular and long interval (Vaario, 1996). Ang (2009) reported that there was no flowering in 2007 and 2008. In addition, *D. beccarii* Dyer posses a recalcitrant seed. According to Tompsett & Krishnapillay (1998), recalcitrant seed shed from the mother plant with very high moisture content which is about 40-60% on a wet weight basis. They die if dried below relatively high moisture content and if they are subject to damage at low temperatures (< 16°C). Thus, the viability of seed in most dipterocarps is short, therefore, once seed is collected, it must be sown immediately or it will be perish (Marzalina, 2002).

Bacteria, viruses and especially fungi also cause loss of seed viability. During seed storage, large quantities in a container and high moisture are propitious condition for fungal development. Two categories of seed fungi that had been identified were the storage fungi and seedborne fungi. The storage fungi include saprophytic fungi growing on the seed testa and seedborne fungi refer to pathogenic fungi that develop from internal part of seed (Apanah & Turnbull, 1998).

Seed derived plants showed wide variations in the field due to genetic variations. This creates problems in forest plantation because seed derived planting stocks are not true-to-types and may not have the superior genotypes from parents trees. Furthermore, natural cycles using seeds will take a relative long time compared to conventional vegetative propagation and micropropagation. Therefore, production of planting stock through seed germination in *D. beccarii* Dyer is not practicable method to obtain large amount of planting stock (Kwan, 2008).

The use of vegetative propagation by cutting is an alternative method of supplying dipterocarpaceae planting stock for artificial regeneration. However, dipterocarp is a species that considered hard to root Momose (1978) and only limited success has been achieved (Rao, 1993). The production planting material by rooting of stem cutting from mature tree of *D. beccarii* Dyer have been studied by Edward (2005) and Jonip (2008) and they reported it was quiet difficult. On the other hand, the plagiotropic growth may exist in shoots from rooted cuttings (Kandasamy *et al*., 2005). Therefore, it is difficult to plan a planting activity for forest plantation by using seeding and conventional method.

2.3 Micropropagation

Plant propagation via *in vitro* culture technique is also termed as micropropagation. The objective of micropropagation is to propagate true-to-type plant, which is clone. Therefore, genetic improvement and conservation of germplasm can be easily achieved by selection of plants with superior genotypes and planting the clonal material of the selected plants (Kwan, 2008).

Tissue culture or *in vitro* culture started from pieces of whole plants, which is called as explants. The correct choice of the explants material can have an important effect on the success of the *in vitro* culture. In mass propagation of woody species, the pathway of plant regeneration through proliferation of shoot tip or axillary bud is commonly followed. However, plants regenerated from axillary bud may exhibit plagiotropic growth, thus shoot tip culture is usually preferred. Kwan (2008) had been studied the organogenesis of shoot by using shoot tips of *D. beccarii* Dyer, however no multiple shoot proliferation was observed

in media that supplemented with cytokinin either they are used singly or in combination with auxin. Moreover, callus that induced from this explants turned black after several months cultured.

There are disadvantage of micropropagation, which is regeneration is often not possible; especially with adult woody plant material. According to Tompsett (1998) propagation of dipterocarps is not easy as there is high rates of cell necrosis that is caused by high resin content that have been observed for some species. However, Vaario (1996) reported that several researchers have been successfully cultured the tissue of some dipterocarps species. In 1993, Smits and Strugcken performed preliminary experiments with three species; *Shorea curtisii, Shorea obtuse* and *Dipterocarpus grandiflorus*. Callus and roots were obtained from *S. curtisii* leaves and nodal segments of *S. obtuse* sprouted axillary buds, however complete plantlets were not obtained. Up to date, Ang (2009) has been successful in induction of root using petiolule as the explant. A 70 % axenic culture had been achieved. However, the problem of browning and contamination by bacteria and fungi still remain as the two major hindrances in micropropagation of *D. beccarii* Dyer.

2.4 Establishment Axenic and Viable Culture

2.4.1 Browning Problem

Phenolics are secondary metabolites that modulate plant development (Arnaldos *et al.,* 2001 as cited in Abdelwahd *et al.*, 2008)**.** However, many authors have observed that phenolics generate toxic compounds in plant tissue culture media (Abdelwahd *et al.,* 2008) that

negatively affect the *in vitro* generation of some tree species (Dibax *et al*., 2005) and crop species (Sharada *et al*., 2003 and Prajapati *et al*., 2003). The phenolic exudates that released from the cut end of explants will oxidized by enzyme polyphenoloxidase (PPO) to the highly toxic quinines (Pingsheng *et al.*, 2009). Oxidized phenolics inhibit enzyme activity and consequently will cause browning of the explants as well as the culture medium and subsequent lethal browning of explants (Laukkanen *et al.*, 1999). The explants eventually die. Browning of media is common especially from tree species and mature tissues from the woody species (Poudyal *et al.*, 2008).

According to Thomas & Ravindra (1999), the released of phenolics affect plant tissue response in shoot induction. However, browning did not affect the growth of root and shoots when explants were cultured in large volume of medium. But in small volume it was lethal (Bhat & Chandel, 1991). Phenolic concentration frequently is affected by several internal and external factors (Ozygit *et al.*, 2007). Stress factors such as drought, water and radiation influence the concentration of phenolics in plants (Kefeli *et al.*, 2003 & Zapprometov *et al.*, 1989). While, according to Lux-Endrich *et al.* (2000), the composition of phenolics in plants influence by some nutrient especially carbohydrate. Ang (2009) reported that as far as the gelling agent was concerned, 'phytagel' caused less browning in shoot-tip explants of *D. beccarii* compared to 'gelrite'.

In the establishment of axenic and viable culture of *D. beccarii* Dyer, seems like browning of culture is currently a greater problem than microbial contamination (Ang, 2009). The strategies and methods therefore should be developed and improved to defeat the harmful

effect of browning attempt to either neutralize or avoid the accumulation of toxic substance in the medium (Bhat & Chandel, 1991). According to several authors, browning in culture can be reduced by one or more of the following measures:

- ✓ Keeping the culture in the dark condition may reduce the browning (Aminah *et al*., 2002 & Helgde & Kulasekaran ,1996).
- ✓ Addition of the activated charcoal (0.5-2 g/L) to the culture medium to absorb undesirable exudates (Helgde & Kulasekaran, 1996). However, the use of activated charcoal is not ideal when the culture medium was added with plant growth regulator or antibiotic as these substances will be also adsorbed by activated charcoal render them become less effective (Kwan, 2008).
- ✓ Carry out the experiment in cold condition. Polyphenol oxidation might be prevented at low temperature (10-15 ºC) and browning was delayed for 2-3 days (Helgde & Kulasekaran, 1996).
- ✓ Choose juvenile explants, or new growth flush during the active growth period (Bhat & Chandel, 1991).
- ✓ Frequent transfer of explants to fresh medium could alleviate browning problem quiet effectively (Kwan, 2008).
- ✓ Inclusion of antioxidants such as citric acid and ascorbic acid in the culture medium, or soaking explants in antioxidant solution prior to inoculation (Titov *et al.*, 2006).
- ✓ Culture in liquid WPM medium could reduced browning and maintain viability of the shoot tip explants. However, the explants began to turn brown after transferred to the

solid media. Consequently, all petiolule explants of *D. beccarii* died at one month after initiation of culture (Ang, 2009).

- ✓ Sealing the cuts ends with paraffin wax (Bhat & Chandel, 1991).
- ✓ Choice of low salt medium and proper growth regulators (Bhat & Chandel, 1991). Although MS and WPM have significant effect on the percentage of bud break and shoot length, but Giridhar *et al.* (2004) reported that WPM was superior to MS and this was true in many medicinal plants culture such as *Cinnamomun caphora*. In addition, MS medium contained high concentration of salt such as KNO_3 which stimulate the production of phenolics (Ang, 2009). At 1998, Vaario had studied the propagation of *Shorea leprosula* which is belonging to Dipterocapaceae. Axillary buds from nodal segments of *Shorea leprosula* were cultured in different media which are WPM, B5, MS, and IS. It was observed that the explants cultured in WPM remained green and showed some axillary bud formation. On the media namely MS, B5 and IS, the explants turn brown. In addition, the highest rate of shoot elongation on shoot-tips is observed in WPM.

2.4.2 Fungal and Bacterial Contamination

Contaminations by fungi, bacteria or yeast present a major challenge for the initiation and maintenance of viable *in vitro* culture which may cause the losses of small number of cultures into the loss of whole batches. These contaminants were particularly dangerous when they are plant pathogens. The problem is further exacerbated when explants material is sourced directly from field-grown plants (Webster *et al.*, 2003). Plant grown in the external environment is always contaminated with micro-organisms and pest. These contaminants are

of mainly confined at the outer surface of the plant and some microbes and viruses may be systemic within the tissues (Edwin, 2008). Therefore, it is extremely difficult controlling the fungal and bacterial contamination of field-grown woody plant material compare to those that grown in greenhouse (Michael & Randall, 1998).

According to Kwan (2008), contamination in *D. beccarii* Dyer was mainly of fungal origin which is actually caused by fungi that naturally present on the surface and natural openings on the explants material. From the previous studies by Tie (2007) and with her personal communication with Jaya Seelan, according to the color of the spores and mycelium, the fungal that grown in *D. beccarii* Dyer culture was identified as *Aspergillus sp.* This fungus was widely recorded on dipterocarps seed (Apanah & Turnbull, 1998). Fungicides such as Benlate (active ingredient is Benomyl) and Topsin M have activity against *Aspergillus sp.* (Amanda, 2002). However, disinfectants will not easily eliminate endogenous contaminants and if disinfectant solutions are too weak, or if the contaminant is located in plant crevices (Norsyarina, 2006).

Thus, it is important to establish an effective disinfection method to generate contamination-free explants for culture. Various types of antimicrobial chemical agents have been tested in plant tissue cultures. They included antibiotics, fungicide and oxidizing biocides such as halogen compounds like chlorine, bromine and iodine (Michael & Randall, 1998).

2.4.2.1 Effects Incoporation of Antibiotics in Culture Media

Antibiotics have been extensively tested for their ability to inhibit or prevent the growth of bacteria in plant cultures. Ideal properties of antibiotics for use in plant tissue culture are, they must be soluble, stable, unaffected by media pH or components, inexpensive, have minimal side effects (no phototoxicity), systemic in plant tissues, has broad spectrum of activity, low chance of bacterial resistance and lethal to microbe**.** Theoretically, all microbial contaminants can be eliminated from plant tissue cultures by one or more antibiotic. However, in reality this is seldom possible (Seckinger & Kenneth, 2004).

There are some limitations when using antibiotic. For example, some antibiotic such as Tetracycline are expensive and some antibiotics is heat labile, phytotoxic to explants, and some bacteria may become resistant toward a particular antibiotic if it is used for a long time. Therefore, care should be taken in disposing media that contained antibiotic. According to (Seckinger & Kenneth, 2004), phytotoxicity of antibiotics were varies between plant species, between genotypes within a species and between plant parts.

Tetracycline is a semi-synthetic antibiotic that produced by bacteria of genus *Streptomyces sp.* which is effective against a wide range of Gram positive and negative bacteria and also mycoplasmas. The concentration that is recommended for woody plant culture is 25-100 µg/ml. Ten percent of stability of Tetracycline will lost in 24 hours if the solution is store in room temperature and if the solution is kept in 5 °C condition, the same percent of stability will lose in 48 hours (Seckinger & Kenneth, 2004).